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13. ABSTRACT *(Maximum 200 Words)*

The focus of this research is to generate and characterize novel human antibody fragments (Fabs) and peptides that bind breast cancer cells. A combinatorial approach is being taken to generate immunoglobulin Fab that recognize a specific determinant on breast cancer cells including the breast cancer-associated Thomsen-Friedenreich (T) glycoantigen. In addition, human Fab and peptides will be generated from combinatorial libraries that bind breast cancer cells, irrespective of defined cell surface markers. The hypothesis that is implicit in these studies is that combinatorial approaches will provide Fab and peptides specific to either defined or ill-defined antigens present on breast cancer cells. The goals of this study are to: 1) generate human IgG Fab that bind specifically to the T antigen expressed on breast cancers from pre-existing Fab, 2) isolate Fab that bind ductal and lobular breast carcinoma cell lines from human combinatorial phage Fab display libraries, and 3) isolate peptides that bind breast carcinoma cell lines from combinatorial phage peptide display libraries. Results suggest phage libraries can yield Fab and scFv that bind to T antigen. Peptides and Fab that recognize lobular and ductal breast cancer cells are presently being sought.

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Combinatorial Approach to the Isolation of Human Antibody Fragments and Peptides to Breast Carcinomas

Final Report

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(4) INTRODUCTION

Continued basic research in breast cancer is of fundamental importance since breast cancer is the leading cause of death in women (1). Breast cancer is known to develop from normal epithelium through several stages. It is not certain exactly when malignancy begins, however "invasive" breast carcinoma with metastatic potential occurs when epithelial cells invade the surrounding stroma (2). Invasive breast carcinoma may be preceded by noninvasive ductal or lobular hyperplasia or carcinoma. Preinvasive lesions as well as invasive breast tumors can often be detected by Mammography. Mammography has not been as reliable for the detection of these lesions in women with dense breast tissue, found commonly in pre-menopausal women. Therefore, a significant percentage of women may go undiagnosed by mammography. Alternative detection, diagnostic, and therapy regimens would be facilitated by reagents that can easily and sensitively detect breast cancer cell markers. The hypothesis that is implicit in these studies is that combinatorial human IgG and peptide libraries will provide molecules specific to antigens present on breast cancer cells. The Fab and peptides obtained will be valuable tools that will expedite basic and applied research into defining the determinants of breast cancer that may also facilitate early detection and diagnosis. The peptides and Fab that bind breast cancer cells, once radiolabeled, may be developed into important new therapeutic or imaging reagents. Novel approaches will be employed to generate new and improved human Fab that specifically recognizes breast cancer cells. Fab immunoglobulin that recognize the well-documented breast cancer-associated Thomsen-Friedenreich (T) glycoantigen will be obtained. In addition, a combinatorial approach will be taken to isolate human Fab against breast cancer cells, regardless of detailed knowledge of target antigens. Combinatorial methods will also be applied for the isolation of small peptides that bind breast cancer cells. Both peptides and Fab that bind breast cancer cells are sought because it is difficult to predict which type of molecule will possess the most ideal properties for a diagnostic or therapeutic.

(5) BODY

The relevance of our proposed research is reflected in our goals of obtaining Fab and peptides that specifically and tightly bind breast cancer cells or cancer antigens. A limited number of murine monoclonal antibodies have been generated by others to the few known breast cancer-associated antigens, although none of them are cell and carcinoma specific. Moreover, the well documented immunogenicity and clearance problems of murine antibodies necessitates the development of 1) specific human Fab for application to human breast cancer and/or 2) the development of small molecule breast cancer-targeting agents. Our progress in the first year is summarized below.

Technical Objective 1: Generate human IgG Fab that bind specifically to the T antigen.

Task 1: Months 1-6: Generation of T Antigen Specific Fab by CDR Random Mutagenesis. Sequence Fab, and generate a database of T-binding sequences.

Research Accomplished: Discussed in year one progress report. Human IgG random CDR combinatorial Fab phage display libraries were screened for Fab that bound T antigen presented as a BSA-T antigen conjugant. After four rounds of affinity selection, individual phage clones were plated out and analyzed for antigen binding activity by filter binding assays. Soluble Fab were produced from clones exhibiting the strongest signal in the filter binding assays. The antigen binding affinities and specificities of the soluble Fab clones were determined by enzyme-linked immunosorbant assay (ELISA). Six Fab DNA clones (9, 17, 22, 52, 53, 54) with the highest ratio of asialofetuin to BSA binding properties were DNA sequenced. Analysis of the H chain sequence revealed that 5 out of the 6 clones had the same H chain DNA sequence. Clone 52 had a different heavy chain sequence but its CDR3 was homologous to the other clones. The L chains of the Fab were all different from each other, however.

Phage #	Sequence
9,17,22,53,54	Val-**Tyr-Cys-Ala-Lys-Met**-Val
52	Tyr-**Tyr-Cys-Ala-Lys-Met**-Arg

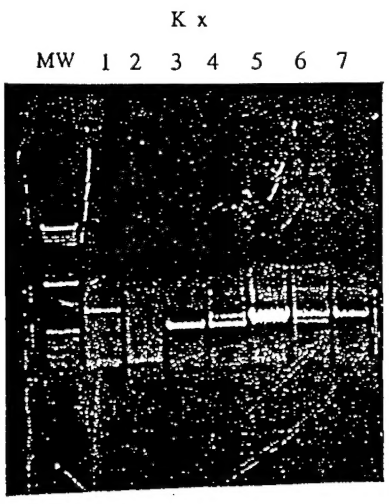
These results suggest a common antigen recognition motif. The isolation of 5 Fab with identical heavy chain sequences but different light chains indicates successful enrichment of Fab during the affinity selection process. Sequencing of more clones confirmed this pattern.

The naive human scFv phage display library was also screened for antibody fragments that bound the carcinoma-associated T antigen. The human scFv library has a diversity of 1 x 10^{10} and several antibody fragments with nanomolar affinities have been isolated from it (6). The phage clones were screened for antigen binding by ELISA. One scFv was expressed in E. coli, purified and re-examined for binding. Its specificity to T antigen appeared to be dependent on the protein rather than the carbohydrate component.

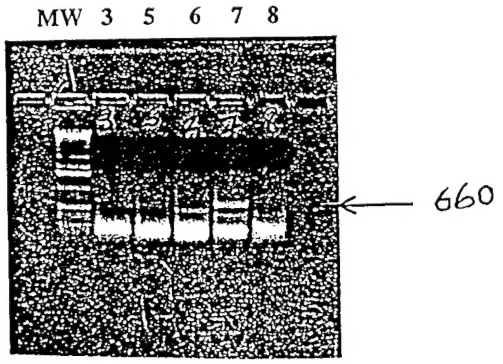
Task 2: Months 6-9: Isolate anti-T IgG from murine hybridomas and isolate human IgG from human sera or phage display libraries.

Research Accomplished: It was originally proposed to isolate IgG hybridomas obtained from collaborator George Springer. Unfortunately, he died recently and all cell lines and hybridomas were bequeathed to a senior investigator who will no longer make them available. As an alternative, we started the production of anti-T hybridomas in mice through injection of cancer cell lines expressing T antigen on their surfaces. Initial screening did not yield T-specific IgG. However, murine anti-T IgG3 was acquired from a new collaborator Kate Rittenhouse-Diakun. A hybridoma cell line producing the anti-T antibody (f11) was obtained and propagated. Cells were harvested and RNA was isolated. Murine IgG and kappa light chain primers were used on generated cDNA to clone the respective Fab genes (Figure 1). Two major PCR products were generated for the light chain and only one set of primers yielded the expected 600-700 base pair fragment of the heavy chain.

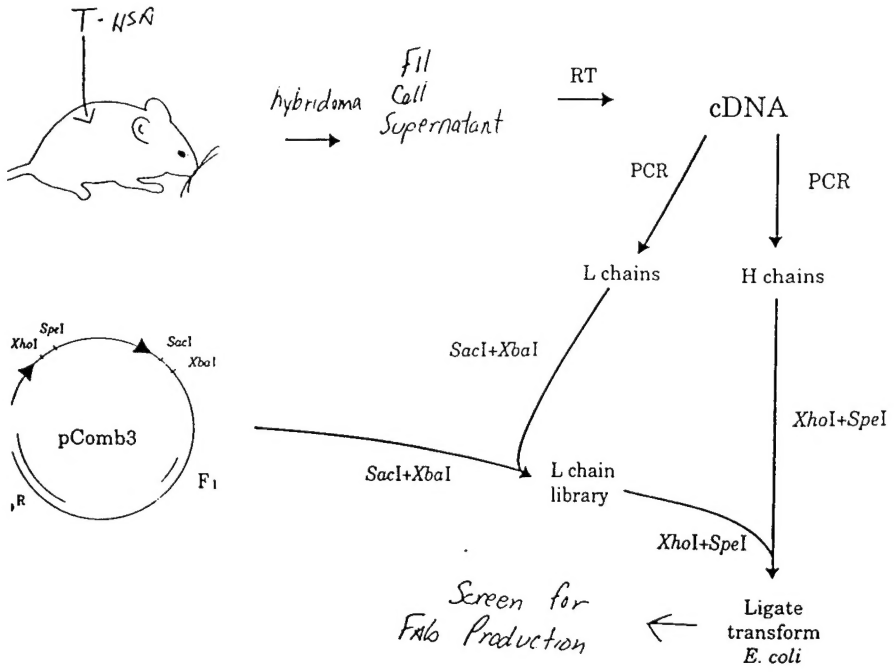
L Chain PCR of F11



H Chain PCR of F11



The PCR products were purified, restriction digested, and sub-cloned into the phagemid expression vector pComb 3 (see Figure 2).



The light and heavy chain DNA and corresponding amino acid sequences were determined. The complementarity-determining regions (CDRs) that dictate binding ability are shown in Figure 3.

Figure 3: Deduced Amino Acid Sequence of F11 Anti-T Antibody CDRs.

VH	CDR1	CDR2	CDR3
F11	TYWMH	FISPNTDYTEYNKFRD	SFIGYNPDF
		(P30) FILP W WYAFSRI	
MK5	KYWMH	YINPSSG YTDYNQKFKG	SAYYRSFDY
DNA-1	SYVMH	YINPYND GTKYNEKFKG	GGYRPYYAMDY

VL			
F11	QASISCRSSQTI	KVSNRFS	FQGSHVPFT
MK5	SASSSVSSRFLH	DTSKLAP	HQWSSYPLT
DNA-1	RASENIYSYLA	NAKTLAE	QHHYGTPLT

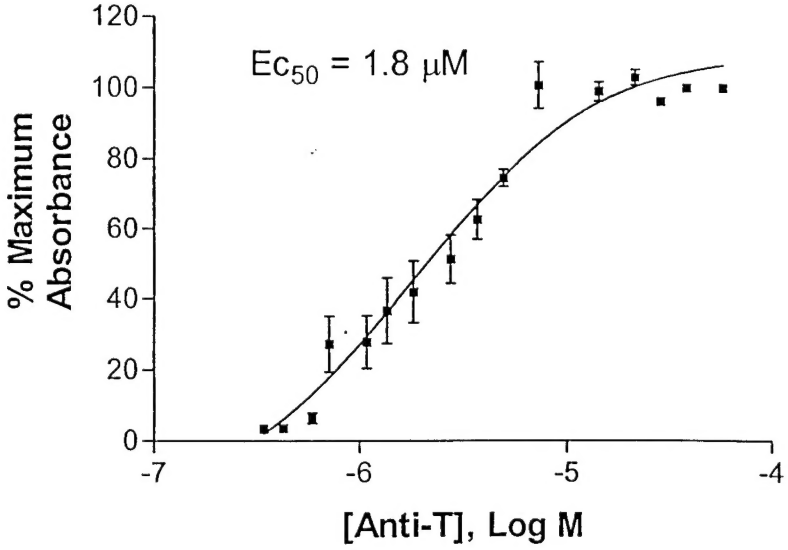
We have continued to attempt expression in E. coli, but so far recombinant f11 appears to be poorly expressed. A light chain switch of another Fab with f11, resulted in improved expression, however the alternate light chain reduced f11 binding to T antigen.

Task 3: Months 9-12: Evolution of a T Antigen-Specific Fab by DNA shuffling. Sequence Fab, express Fab in *E. coli*.

Research Accomplished: We transplanted the murine variable region of anti-T f11 into a murine Fab that expresses well. The Fab expressed to measurable levels in E. coli, however the specificity for T antigen was markedly reduced.

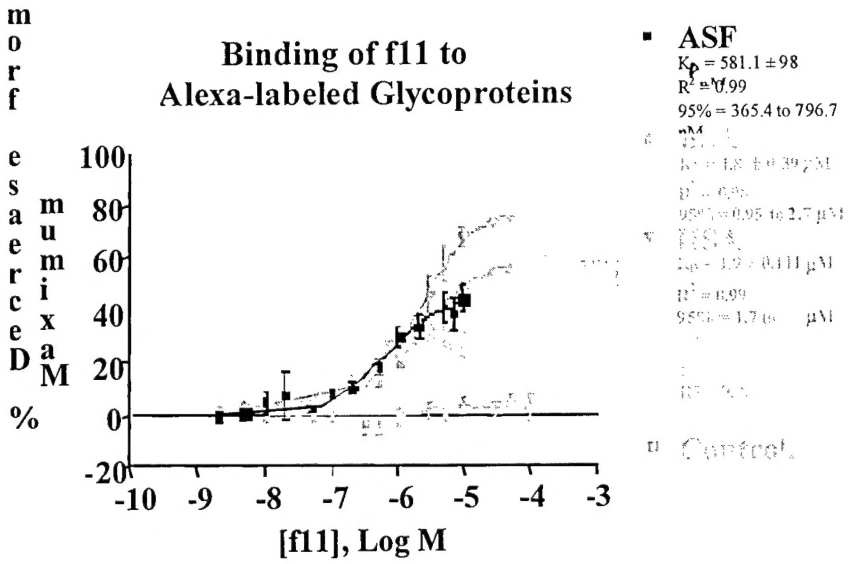
Task 4: Months 12-18: Purify Fab using affinity chromatography and examine binding to T antigen by ELISA, immunofluorescence and fluorescence titration.
Research Accomplished: We expressed four anti-T soluble scFv in *E. coli*. The scFv were secreted into both the media and the periplasm. Soluble scFv were produced from phage clones exhibiting the best ELISA results. The assays yield indicated the scFv were not specific for T antigen and could bind other antigens as well. We have spent months 12-18 purifying anti-T f11 antibody to homogeneity. The Fab recognizes the GalB1-3GalNac moiety of T and not other carbohydrates tested. ELISA results indicate the antibody has a high nanomolar affinity to T displayed on asialofetuin (ASF). See Figure 4.

Figure 4: ELISA of Anti-T Antibody Binding to ASF



We performed fluorescence quenching titration with the Fab to determine an accurate affinity constant. Studies from our laboratory as well as others have verified the effectiveness of solution-phase tryptophan fluorescence quenching assays to accurately define binding parameters of anti-carbohydrate Fab and Abs. The fluorescence spectroscopic methods are based on the high sensitivity and accuracy of the intrinsic fluorescence from tryptophan residues in the Ab molecule and will monitor the change in tryptophan fluorescence (quenching) upon ligand binding. Fluorescence titration experiments ideally measure a range of dissociation constants from 10^{-9} to 10^{-5} M. The K_d of liganded anti-T Fab f11 was thought to be in the high nanomolar range, so it would be an ideal candidate for these studies. The titrations were performed using a SLM 8100 spectrofluorimeter. Varying amounts of ASF and pure T antigen were added to a fixed Fab concentration. Protein fluorescence was measured with 292 nm excitation and 346 nm emission. The K_d was determined from binding curves derived from the titrations using a single binding-site curve fitting procedure. From the titration experiments, a Kd of 500nM was obtained.

Thus far, quenching data yields a similar dissociation constant to that derived from ELISA.



Fluorescence Quenching Titration of Anti-T f11 to Glycoconjugates

Technical Objective 2: Isolate Fab that bind ductal and lobular breast carcinoma cell lines from human combinatorial Fab display libraries.

Task 1: Months 1-2: Obtain cell lines, grow cells, make frozen stocks and extracts.
Research Accomplished: Discussed in year one progress report. MDA-MB 435, MDA-MB-468 - lobular breast cancer cell lines, Hs578T a ductal cancer cell line, and Hs578Bstneg a normal ductal cell line were purchased.

Task 2: Months 3-12: Affinity select phage Fab libraries against cell lines.
Research Accomplished: We performed affinity selections and sequenced a few resulting Fab. Many of the Fab had the same amino acid sequence as in control cell lines such that we believe the selections were not generating cancer specific Fab.

Task 3: Months 13-16: Identify clones that bind cell lines using colony hybridization.
Research Accomplished: We have analyzed fifty clones to see if they bind specifically to breast cancer cells. So far phage that bind only to breast cancer cell lines and not normal cell lines have not been obtained. We decided this last year to focus on peptides that bind T, since the f11 Fab is specific for T, and appears to be a good candidate for future studies.

Task 4: Months 17-24: Sequence Fab genes, express Fab and purify using affinity chromatography, and characterize binding using immunofluorescence and fluorescence titration.
Research Accomplished: We sequenced twenty representative phage inserts from the different cell line panning experiments. A general consensus sequence was not defined. It may be necessary to utilize more stringent conditions or perform more rounds of selection to increase our chances of finding consensus motifs. ELISA assays with the twenty clones did not yield specific binders for only cancer cells. We have run out of time on this project to analyze more clones.

Technical Objective 3: Isolate peptides that bind breast carcinoma cell lines from combinatorial phage peptide display libraries.

Task 1: Months 25-28: Affinity select phage peptide libraries against cell lines.
Research Accomplished: Each of the cell lines was selected against a random 15mer phage peptide display library. Four rounds of affinity selection have been completed. We performed this aim early because it took longer to garner antibody for technical objective 1. This objective does not rely on the anti-T antibody.

Phage (approximately 10^{10}-10^{12} virions) from a phage display library displaying random 15-mer peptides on its pIII coat protein, were added to each dish of cells containing growth media. Affinity selection was performed similar to that for the Fab libraries outlined in Task 2, Objective 2.

Following the wash stages, specifically bound phage were eluted with resuspension and gentle agitation. A small portion of the eluted phage was used to produce serial dilutions of the phage which were subsequently used to infect K91 Blue Kan *E. coli* cells. Cells were then plated on agar plates containing kanamycin and tetracycline - K91 Blue Kan cells carry resistance to kanamycin and the phage that infect the cells will confer tetracycline resistance to those cells. A portion of the first round eluted phage was used to infect *E. coli* cells which were subsequently used to produce phage particles to be used as the input phage for biopanning round #2. We have finished four rounds of selection with MDA-MB 435 and MDA-MB-468 breast cancer cell lines. Fifty clones from this round were further analyzed. Phage from the clones were isolated and ssDNA was prepared. The DNA was first single lane sequenced to identify unique clones, and unique clones were subjected to standard four lane dideoxysequencing.

The following peptide sequences were found in the selected population after four rounds of panning:

```
Peptide 1:QADGPNSVVRPFTLT
Peptide 2:FALRPPGPNRPFTAA
Peptide 3:PILFDGTARGMLVRS
Peptide 4:GSFGSTGTGRSVRI
Peptide 5:TGLLFPSFEWTYAQF
Peptide 6:HSAVGFAWPYRGLVL
Peptide 7:EVPRLSLLAVSLVAN
Peptide 8:PDRWFREMASMVYRS
Peptide 9:GDGDLVRHVVCVNGC
```

Task 2: Months 29-30: Characterize binding using immunofluorescence and fluorescence titration.
Phage displaying these peptides were first analyzed using immunoblotting to determine which peptides might bind specifically to T antigen. Immunoblots indicated that peptides 1, 4, 7, and 9 may bind T antigen. We performed immunofluorescence with the phage using breast cancer cell lines and control cells not expressing T antigen. The immunofluorescence procedures relied on an anti-M13 phage antibody for detection. It was noted that a high background was present in control experiments, and it was decided to further analyze binding of the peptides without interference of the phage.

Task 3: Months 31-36: Chemically synthesize selected peptides and determine binding properties to breast cancer cell lines.

The peptides 1, 4, 7, and 9 were chemically synthesized and analyzed for binding using both immunofluorescence and fluorescence quenching. The peptides all bound to some extent to breast cancer

cells, and at high concentration (micromolar) binding was detected in fluorescence quenching assays. At low concentrations, peptide 7 appeared to bind the most selectively to T antigen-bearing cell lines including MDA-435, DU-145 (prostate), and ovarian cancer cell lines. The affinity of this peptide for T was estimated to be 5uM. Further studies will analyze the utility of these peptides in breast cancer cell imaging.

(6) KEY RESEARCH ACCOMPLISHMENTS

* Affinity Selection of Random HCDR Fab and scFv libraries Against T Antigen.
* Determine DNA and Amino Acid Sequence of 5 Fab clones.
* Purify anti-T scFv and Examine Binding by ELISA.
* Purify anti-T monoclonal antibody f11.
* Determine affinity of anti-T antibody to T-containing asialofetuin using ELISA and fluorescence quenching.
* Isolate mRNA from f11 hybridoma and generate cDNA.
* Clone via PCR the heavy and light chain Fab genes of f11.
* Determine the DNA and encoded amino acid sequence of the f11 Fab.
* Subclone the heavy and light chains of f11 into E. Coli expression vector pComb3,
* Screen Antibody and Peptide libraries for Molecules that Bind Lobular and Ductal Breast Cancer Cell Lines.
* Study role of T antigen in cell adhesion.
* Isolate peptides that bind T antigen and study their binding characteristics

(7) REPORTABLE OUTCOMES

* Clone and sequence anti-T IgG3 heavy and light chains.
* DOD Breast Cancer Postdoctoral Fellowship Awarded to Mark Meighan, a post-doctoral fellow in my laboratory.
* Attend ERA of Hope meeting in Atlanta and present poster or our work.
* Co-author manuscript on the role of T antigen in adhesion. "Glinsky, V.V., Huflejt, M.E., Glinsky, G.V., Deutscher, S.L., and Quinn, T.P. (2000) Effects of T Antigen Specific Peptide P-30 on β-Galactoside Mediated Homotypic Aggregation and Adhesion to the Endothelium of MDA-MB-435 Human Breast Carcinoma Cells. Cancer Res. 60, 2584-2588."

* Co-author manuscript on the role of T antigen and Galectin 3 in metastasis. "Glinsky, V.V., Glinsky, G.V., Rittenhouse-Olsen, K., Huflejt, M.E., Glinskii, O.V., Deutscher, S.L., and Quinn, T.P. (2001) The Role of Thomsen-Friedenreich Antigen in Adhesion of Human Breast and Prostate Cancer Cells to the Endothelium. Cancer Res. 61, 4851-4857.

(8) CONCLUSIONS

Alternative detection, diagnostic, and therapeutic approaches are needed to help reduce the morbidity and mortality of breast cancer. Our approach is to employ combinatorial human Ig and peptide libraries to generate molecules specific to antigens present on breast cancer cells. The Fab and peptides will be valuable tools that will expedite basic and applied research into defining the determinants of breast cancer, that once radiolabeled, may facilitate early detection and diagnosis. Human IgG random CDR combinatorial Fab phage display libraries and more diverse scFV libraries were screened for Fab that bound the breast cancer-associated T antigen presented as a BSA-T antigen conjugant or asialofetuin. Numerous Fab and scFv-displaying phage were selected in this procedure and analyzed for binding to T antigen using ELISA. Most of the scFv did not bind specifically to the T epitope. We have obtained a new anti-T monoclonal antibody that we have determined binds specifically to the T carbohydrate. The H and L chain genes have been cloned using a diverse set of immunoglobulin primers. The DNA and encoded sequence of the recombinant Fab suggests the anti-T antibody to be rather unique. Its heavy chain is of a rare subclass. The recombinant Fab will be expressed in E. coli and analyzed further (5). We have shuffled CDRs of a non-specific Fab in the hopes of converting it to a T-binding antibody. These problems were addressed through the simultaneous screening of peptide libraries for molecules that bind T antigen or breast cancer cells since peptides may be as or more valuable as diagnostic or therapeutic agents. Peptides were isolated that bind T antigen are are currently being analyzed.

"So What Section"

-Recombinant antibody fragments that bind T antigen may be useful reagents in breast cancer diagnosis, detection, or localization.
-Recombinant antibody fragments can be engineered to contain a radiometal chelation sequence such that radiolabeling with Re186/188 can yield radiopharmaceuticals that could destroy cancer cells.
-Recombinant antibody fragments that bind ductal or breast cancer cells may be used to identify new breast cancer antigens.
-Recombinant antibody fragments that bind ductal or breast cancer cells may be used to differentiate ductal from lobular breast cancer which can assist in developing appropriate therapeutic treatment regimens.
- Peptides that bind breast or ductal breast cells may be used to differentiate ductal from lobular breast cancer which can assist in developing appropriate therapeutic treatment regimens. Peptides may be tolerated in higher doses in patients than antibodies.

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(10) PERSONNEL

Personnel receiving pay:

Susan Deutscher, PI.
Linda Landon, Post-doctoral fellow
Brittany Cone, technician

APPENDIX

Journal Publications

Glinsky, V.V., Huflejt, M.E., Glinsky, G.V., Deutscher, S.L., and Quinn, T.P. (2000) Effects of T Antigen Specific Peptide P-30 on β-Galactoside Mediated Homotypic Aggregation and Adhesion to the Endothelium of MDA-MB-435 Human Breast Carcinoma Cells. Cancer Res. 60, 2584-2588.

Glinsky, V.V., Glinsky, G.V., Rittenhouse-Olsen, K., Huflejt, M.E., Glinskii, O.V., Deutscher, S.L., and Quinn, T.P. (2001) The Role of Thomsen-Friedenreich Antigen in Adhesion of Human Breast and Prostate Cancer Cells to the Endothelium. Cancer Res. 61, 4851-4857.

[CANCER RESEARCH 60, 2584–2588, May 15, 2000]

Advances in Brief

Effects of Thomsen-Friedenreich Antigen-specific Peptide P-30 on β-Galactoside-mediated Homotypic Aggregation and Adhesion to the Endothelium of MDA-MB-435 Human Breast Carcinoma Cells[1]

Vladislav V. Glinsky, Margaret E. Huflejt, Gennadi V. Glinsky, Susan L. Deutscher, and Thomas P. Quinn[2]

Cancer Research Center [V. V. G.] and Department of Biochemistry [V. V. G., S. L. D., T. P. Q.], University of Missouri, Columbia, Missouri 65211; La Jolla Institute for Allergy and Immunology [M. E. H.], San Diego, California 92121; and Sidney Kimmel Cancer Center and Metastat, Inc. [G. V. G.], San Diego, California 92121

Abstract

Both the ability of malignant cells to form multicellular aggregates via homotypic or heterotypic aggregation and their adhesion to the endothelium are important if not critical during early stages of cancer metastasis. The tumor-associated carbohydrate Thomsen-Friedenreich antigen (T antigen) and β-galactoside binding lectins (galectins) have been implicated in tumor cell adhesion and tissue invasion. In this study, we demonstrate the involvement of T antigen in both homotypic aggregation of MDA-MB-435 human breast carcinoma cells and their adhesion to the endothelium. The T antigen-specific peptide P-30 (HGRFILPWWYAFSPS) selected from a bacteriophage display library was able to inhibit spontaneous homotypic aggregation of MDA-MB-435 cells up to 74% in a dose-dependent manner. Because T antigen has β-galactose as a terminal sugar, the expression profile of β-galactoside-binding lectins (galectins) in MDA-MB-435 cells was studied. Our data indicated the abundant expression of [^{35}S]methionine/cysteine-labeled galectin-1 and galectin-3 in this cell line, which suggested possible interactions between galectins and T antigen. As revealed by laser confocal microscopy, both galectin-1 and galectin-3 also participate in the adhesion of the MDA-MB-435 cells to the endothelium. We observed the clustering of galectin-3 on endothelial cells at the sites of the contact with tumor cells, consistent with its possible interaction with T antigen on cancer cells. The galectin-1 signal, however, strongly accumulated at the sites of cell-cell contacts predominantly on tumor cells. The T antigen-specific P-30 significantly (50%) inhibited this adhesion, which indicated that T antigen participates in the adhesion of MDA-MB-435 breast cancer cells to the endothelium. The ability of synthetic P-30 to inhibit both the spontaneous homotypic aggregation of MDA-MB-435 cells and their adhesion to the endothelium (>70 and 50%, respectively) suggests its potential functional significance for antiadhesive therapy of cancer metastasis.

Introduction

Understanding the molecular underpinnings of cancer metastasis is an important goal of modern cancer research. Metastasis is a multistep process involving many cell-cell and cell-extracellular matrix interactions. Several of these steps include interactions between cell surface molecules such as carbohydrates, lectins, and extracellular matrix proteins participating in cell-cell recognition and adhesion (1, 2). Whereas the initial steps of metastasis include detachment of malignant cells from the primary tumor and migration into the circulatory system, subsequent steps involve malignant cells adhering to each other (homotypic aggregation) or to host cells (heterotypic adhesion; Refs. 3–6) to form multicellular aggregates. Eventually, the circulating tumor cells bind to capillary endothelial cells and to exposed basement membrane proteins, which results in the formation of secondary tumor sites. Recent observations by Al-Mehdi *et al.* (7) indicate the critical role of adhesion of the cancer cells to the vascular endothelium in this process. They demonstrated that only endothelium-attached rather than extravasated cancer cells are capable of giving rise to hematogenous cancer metastases (7). It has been suggested that tumor cell adhesion is, in part, mediated by specific interactions between cell surface lectins and carbohydrates present on glycoproteins, glycolipids, and glycosaminoglycans (2, 4, 8, 9).

There has been a tremendous surge in research to characterize the roles of cell surface carbohydrate structures in cell-cell communication as mediators of tumor cell proliferation, adhesion, and metastasis. Alterations in cell surface carbohydrate structures of cancer cells are postulated to effect normal cellular interactions and have been shown to facilitate tumor cell colonization and metastasis (2, 3, 8). One such cancer-associated carbohydrate antigen, the T antigen,[3] has been the focus of much research into its role in tumor cell adhesion and metastasis (8). The immunodominant portion of the T antigen is the terminal Galβ1 → 3GalNAc carbohydrate moiety (5). Cryptic, covalently or structurally masked and nonimmunoreactive, T antigen is present on the surfaces of healthy cells in most tissues. It is, however, exposed and immunoreactive on most human carcinomas and T-cell lymphomas (8, 10). T antigen has been proposed to be involved in tumor cell adhesion and tissue invasion. The existence of T antigen-mediated cell adhesion between highly metastatic murine lymphoma cells and hepatocytes is supportive of a role for this cell surface carbohydrate structure in the metastatic process (6). Large quantities of T antigen have been detected on the outer surface membranes of human breast carcinomas, which makes it an attractive target for the development of tumor diagnostic and therapeutic agents (10, 11). In our laboratory, several peptides that bind T antigen have been affinity-selected from a 15-amino-acid-random-peptide bacteriophage display library and characterized for their binding affinities and specificities (12, 13). One of the peptides, P-30, has been shown to selectively bind several cancer cell lines that display T antigen on their surfaces including MDA-MB-435 human breast carcinoma cells. It was also found to efficiently inhibit asialofetuin-induced homotypic aggregation of B16-F1 murine melanoma cells (13). We hypothesized that if T antigen mediates spontaneous homotypic aggregation of breast cancer cells, then a T antigen-binding peptide may likewise inhibit this aggregation. In this study, we demonstrate that T antigen accumulates at the sites of cell contact in multicellular aggregates of MDA-MB-435 human breast carcinoma cells, which suggests the

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[3] The abbreviations used are: T antigen, Thomsen-Friedenreich antigen; HUVEC, human umbilical vein endothelial cell; PNA, peanut agglutinin; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; Cy5, *N,N′*-biscarboxypentyl-5,5′-disulfonatoindodicarbocyanine.

involvement of T antigen in spontaneous aggregation. Indicative of the participation of T antigen in homotypic aggregation is the ability of T antigen-specific P-30 to significantly (>70%) inhibit this aggregation in a dose-dependent manner.

Cell type-specific carbohydrates facilitate cell-cell communication through selective interactions with carbohydrate-binding proteins, including cell surface lectins (1). The early works of Dr. A. Raz and colleagues [Meromsky *et al.* (4) and Raz and Lotan (9)] suggest an important role of soluble β-galactoside-specific lectins (galectins) in cancer cell adhesion and metastasis. Because the terminal residue of T antigen is β-galactose, one can reasonably suggest its possible interactions with members of the β-galactoside binding lectin family. Therefore, we studied the expression profiles of galectins, namely galectin-1, galectin-3, and galectin-4, in MDA-MB-435 cells. Our data indicated the abundant expression of ^{35}S-labeled galectin-1 and galectin-3 but not galectin-4 in these cells, which suggested a potential interplay of T antigen with galectins, most likely with galectin-3.

Both galectin-1 and galectin-3 appear to participate in the adhesion of the MDA-MB-435 cells to a monolayer of human endothelial cells as revealed by laser confocal microscopy. We observed the accumulation of the galectin-3 on endothelial cells at the sites of their contact with cancer cells, which would be supportive of possible interactions between T antigen and galectin-3. The T antigen-specific P-30 peptide was able to inhibit this adhesion by up to 50%.

The results presented in this paper demonstrate that β-galactoside-mediated, in particular T antigen-mediated, cell-cell interactions are important components of both the spontaneous homotypic aggregation of the MDA-MB-435 human breast carcinoma cells and their adhesion to the endothelium. The ability of P-30 to inhibit T antigen-mediated tumor cell aggregation and adhesion highlights its potential functional significance for antiadhesive therapy of cancer metastasis.

Materials and Methods

Cell Lines and Cultures. The MDA-MB-435 human breast carcinoma cell line, originally isolated from the pleural effusion of a patient with breast carcinoma, was kindly provided by Dr. Janet E. Price, M. D. Anderson Cancer Center, Houston, TX. This cell line was selected for our study because it was found to be highly metastatic in nude mice from mammary fat-pad tumors as well as on i.v. inoculation *in vivo* (14, 15) and exhibited superior aggregation and survivability *in vitro* compared with the other lines tested (16). Tumor cells were maintained in 5% CO_2/95% air at 37°C in a humidified incubator in tissue culture flasks as a monolayer culture using RPMI 1640 supplemented with L-glutamine, 10% fetal bovine serum, sodium pyruvate, and nonessential amino acids.

HUVECs pooled from multiple isolates were purchased from Cascade Biologicals, Inc. (Portland, OR). The cultures were free of HIV-1, Hepatitis B and C viruses, *Mycoplasma*, bacteria, yeast, and fungi. The cells were positive for the DiI-acetylated low density lipoprotein uptake and expression of von Willebrand factor and CD31 but not for the α-actin expression. The HUVECs were maintained on plastic as a monolayer culture in a humidified incubator in 5% CO_2/95% air at 37°C. The basal Medium 200 (Cascade Biologicals), supplemented with low serum growth supplement containing fetal bovine serum (2% v/v final concentration), hydrocortisone, human fibroblast growth factor, heparin, and human epidermal growth factor, was used. The cells at population doublings of approximately 8–12 were used for the adhesion experiments.

Peptide Synthesis and Purification. T antigen-binding peptide P-30 (HGRFILPWWYAFSPS) and control peptide (RRLLFYKYVYKRYRAG-KQRG) were chemically synthesized on the Applied Biosystems peptide synthesizer 431A using *N*-(9-fluorenyl)methoxycarbonyl-based chemistry and purified to homogeneity on a C-18 reverse-phase high-performance liquid chromatography column (ISCO Corp.).

Antibodies. A rabbit polyclonal anti-galectin-1 antiserum was a generous gift from Dr. Douglas W. N. Cooper (University of California, San Francisco, CA). A rat monoclonal anti-galectin-3 (anti-Mac-2) antibody (17) was used as described previously (18). Rabbit anti-galectin-4 serum was raised using the COOH-terminal domain of rat intestinal galectin-4 as immunogen as described previously (19). Cy5-conjugated goat antirabbit IgG was purchased from Jackson Immuno Research Laboratories (West Grove, PA). Goat Texas Red-conjugated antirat antibody was purchased from Molecular Probes (Eugene, OR).

Cytochemical Analysis of T Antigen. The cytochemical analysis of T antigen was performed using PNA lectin-horseradish peroxidase conjugate, and subsequent color reaction was performed with diaminobenzidine tetrahydrochloride. The direct binding of T antigen-specific PNA lectin to MDA-MB-435 human breast carcinoma cells was performed as described previously (13) with one minor modification. After dissociation of cells from the plastic and before fixing them with 2% formaldehyde-PBS solution and placing on a microscope slide, the cells were allowed to aggregate for 30 min in serum-free RPMI 1640 at 37°C.

Cell Aggregation Assay. A homotypic aggregation assay of MDA-MB-435 cells was performed as previously described (4, 20). The only modification was made for the samples prepared for the cytological analysis of T antigen. In these experiments, cancer cells were allowed to aggregate for 30 min instead of 1 h to avoid formation of excessively large multicellular aggregates.

Analysis of Galectins Expression in MDA-MB-435 Cells. The metabolic [^{35}S]methionine/cysteine labeling of galectins followed by affinity purification on lactosyl-Sepharose and separation by SDS-PAGE was performed exactly as described previously (21). Densitometry of SDS-PAGE of the purified galectins was used to assess the relative amounts of each galectin. On the basis of the absolute yield of lactosyl-Sepharose purified galectins and the estimated volume of the confluent monolayer of MDA-MB-435 cells, the approximate molar concentrations of galectins 1, 3, and 4 were calculated as described previously (21).

Adhesion to the Endothelium. HUVECs were grown to confluence directly on microscope slides using the four-well Lab-Tec II chamber slide system (NalgeNunc, Naperville, IL). Twenty-four h before the adhesion experiment, the endothelial cell cultures were switched to quiescence medium (Medium 200 without low serum growth supplement), and MDA-MB-435 human breast carcinoma cells were prelabeled with 5 μg/ml solution of DiI (Molecular Probes) in serum-free RPMI 1640 for 60 min at 37°C. Immediately before the experiment, cancer cells were dissociated from plastic using a nonenzymatic cell dissociation reagent (Sigma, St. Louis, MO), and pipetted to produce a single-cell suspension. DiI-labeled breast carcinoma cells [5×10^4 cells per chamber in 2.5 ml of serum-free medium supplemented with various concentrations of P-30 (0 to 0.1 mg/ml) or control peptide] were added to the monolayer of the endothelial cells. The chambers were sealed with adhesive tape while ensuring that no air bubbles were trapped. The cells were allowed to adhere for 1 h at 37°C, after which the chambers were inverted and left upside down for 30 min to allow sedimentation of nonadhered cells. At the end of the incubation, the medium was drained while chambers were still upside-down. Samples were gently rinsed with PBS, fixed for 30 min in 2% formaldehyde solution in PBS, mounted under cover glass, and examined by fluorescent microscopy. Four random fields in each well were photographed at ×250, and the total number of adhered cells in every field was counted. The assay was performed in quadruplicate for each concentration of the peptides tested.

Laser Scanning Confocal Microscopy. The samples for laser scanning confocal microscopy were prepared exactly as described above in "Adhesion to the Endothelium," except that the cancer cells used in these experiments were not prelabeled with DiI, and samples were fixed (but not permeabilized) in 2% formaldehyde solution in PBS for 24 h. The antibodies against galectins-1, -3, and -4 were used as described previously (20). The goat Texas-Red-conjugated antirat antibody and Cy5-conjugated goat antirabbit IgG were used as secondary antibodies at a dilution of 1:100. The laser scanning confocal microscopy was performed with a Bio-Rad MRC 600 confocal system. The RHS and YHS blocks were used to detect fluorescence emitted by Cy5 and Texas Red respectively. The Z stacks were prepared by obtaining serial sections with 0.5-μm increments and analyzed in orthogonal projections (Y-Z and X-Z sections) using the MetaMorph Imaging System software (Universal Imaging, Hallis, NH).

Results and Discussion

Involvement of T Antigen in Homotypic Aggregation of MDA-MB-435 Human Breast Carcinoma Cells. Multicellular aggregate formation is an important feature of metastatic cancer cells directly correlating with their increased survival potential *in vitro* (20) and metastatic propensity *in vivo* (22). The cancer-associated T antigen has been implicated in tumor cell adhesion through carbohydrate-lectin interactions (6, 23). We previously reported the expression of large quantities of T antigen on the surface of MDA-MB-435 cells that was confirmed by the binding of T antigen-specific PNA lectin (13). In this study, we investigated the role of T antigen in homotypic aggregation of the MDA-MB-435 breast cancer cells. Tumor cells collected from subconfluent (70–80%) cultures were allowed to form multicellular aggregates as described in "Materials and Methods." The direct binding of T antigen-specific PNA lectin, conjugated to horseradish peroxidase followed by color reaction with diaminobenzidine tetrahydrochloride, was used to visualize T antigen. The cytochemical analysis of the samples that contained multicellular aggregates revealed significant accumulation of T antigen at the sites of cell contacts (Fig. 1*A*), which suggested participation of T antigen in homotypic aggregation of MDA-MB-435 breast carcinoma cells. Consistent with this is the fact that the addition of different concentrations of synthetic T antigen-specific peptide, P-30 (HGRFILPWWYAFSPS), inhibited homotypic aggregation of MDA-MB-435 cells in a dose-dependent manner (Fig. 1*B*). A maximal inhibitory effect (>70%) was achieved at a peptide concentration of 0.1 mg/ml. The control peptide (RRLLFYKYVYKRYRAGKQRG), which does not interact with T antigen (13), failed to inhibit homotypic aggregation of MDA-MB-435 cells (Fig. 1, *C–E*). These findings, as well as the previously reported ability of P-30 to inhibit asialofetuin-mediated aggregation of mouse melanoma cells (13), suggest that the effect of P-30 on homotypic aggregation of MDA-MB-435 cells is T antigen-specific.

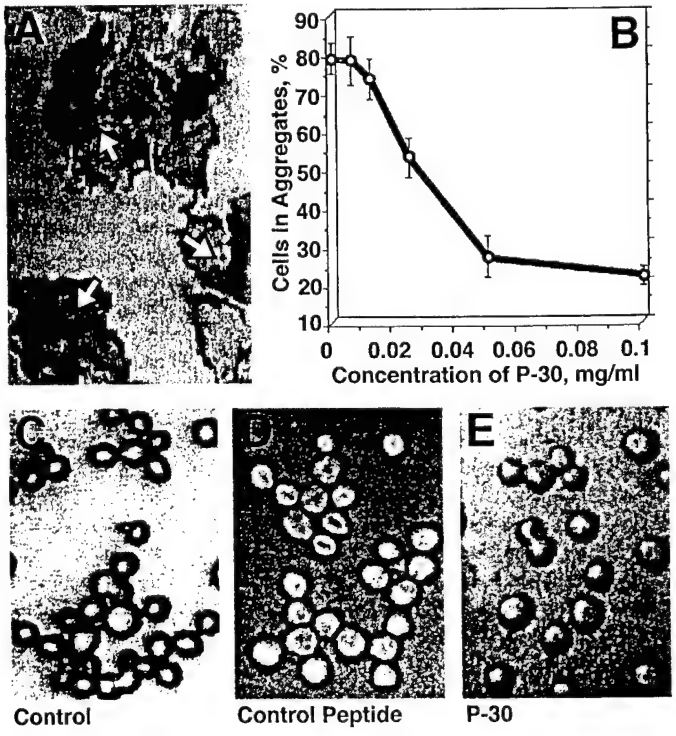

Fig. 1. *A*, direct binding of T antigen-specific PNA to the MDA-MB-435 human breast carcinoma cells. *Arrows*, accumulation of T antigen at the sites of the cell-cell contact. *B*, dose-dependent inhibition of spontaneous homotypic aggregation of the MDA-MB-435 human breast carcinoma cells by T antigen-specific peptide P-30. *C–E*, inhibition of spontaneous homotypic aggregation of MDA-MB-435 human breast carcinoma cells by 0.1 mg/ml of synthetic P-30 (*E*) but not by the same concentration of the control peptide (*D*) compared with the control (*C*).

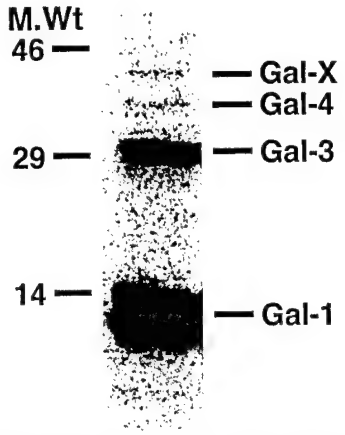

Fig. 2. [^{35}S]Methionine/cysteine-labeled expression profile of β-galactoside-specific lectins (galectins) in MDA-MB-435 human breast carcinoma cells. *On the left*, the position of the molecular weight markers (*46*, *29*, and *14*, for M_r 46,000, 29,000, and 14,000, respectively).There is an abundant expression of galectin-1 and galectin-3 compared with a very weak expression of galectin-4. The additional weak band corresponding to the molecular weight of approximately M_r 40,000 suggests a low level of expression of an unidentified soluble β-galactoside-specific lectin (*Gal-X*).

Expression of β-Galactoside-specific Lectins (Galectins) on MDA-MB-435 Cells. Because T antigen (Galβ1 → 3GalNAc) has β-galactose as a terminal sugar, it is likely that T antigen-mediated interactions may involve the participation of β-galactoside-specific lectins (galectins). Thus, we studied the expression profile of the galectins, namely galectin-1, galectin-3, and galectin-4 in MDA-MB-435 cells. Metabolic [^{35}S]methionine/cysteine labeling followed by affinity purification on lactosyl-Sepharose and separation by SDS-PAGE was used to isolate galectins and characterize their expression in this cell line. The results of these experiments (Fig. 2) identified galectin-1 as a major β-galactoside-specific lectin expressed in MDA-MB-435 breast carcinoma cells. The estimated molar concentrations of galectins-1, -3, and -4 in MDA-MB-435 cells were in the range of 1–3 μM, 100–500 nM, and 10–50 nM, respectively. The presence of the additional minor band corresponding to a molecular weight of M_r ~40,000 (Fig. 2) suggests the weak expression of another, yet unidentified, soluble lactose-binding lectin (Gal-X). Human galectin-3 displays a 20-fold higher specific activity in binding to the Galβ1→3GalNAc disaccharide than galectin-1 (24). Thus, galectin-3 is most likely to interact with T antigen. Previously reported inhibition by T antigen-specific P-30 of asialofetuin-mediated aggregation of B16-F1 cells (13), known to be galectin-3-dependent (25), is also supportive of this interaction. The analysis of β-galactoside-binding lectins in 11 other human breast carcinoma cell lines established from pleural or ascitic effusions revealed similar galectin expression profiles,[4] which suggests that galectin-1 and galectin-3 overexpression is a phenomenon frequently occurring in metastatic breast cancer.

Adhesion of MDA-MB-435 Breast Carcinoma Cells to the Endothelium. Both galectin-1 and galectin-3 were found to be expressed on the endothelium of various origins in different species including humans (26). Galectin-1 was also shown to participate in murine RAW117-H10 large-cell lymphoma cell adhesion to liver microvessel endothelial cells (26), and galectin-3 was suggested to be, at least in part, responsible for the preferential adhesion of prostate cancer

[4] B. Lundin-Jensen, M. Jazayeri, A. Ponce, F-T. Liu, P. Bryant, and M. E. Huflejt. Galectins in human breast cancer cell lines established from various stages of the breast disease, submitted for publication.

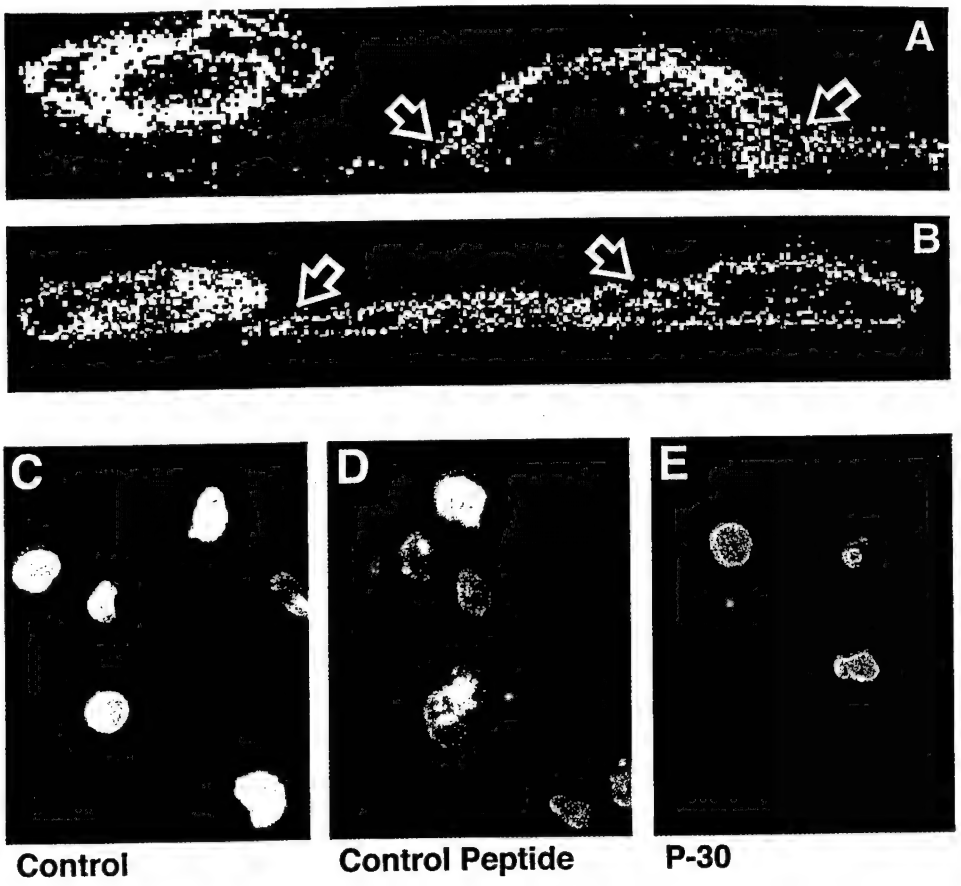

Fig. 3. Involvement of the galectin-1 (*A*) and galectin-3 (*B*) in the adhesion of the MDA-MB-435 human breast carcinoma cells to the monolayer of human umbilical endothelial cells as revealed by laser confocal microscopy. The X-Z sections shown were obtained as described in "Materials and Methods" using a ×60 lens. The images were pseudocolored (*green*, galectin-1; *red*, galectin-3). *Arrows*, clustering toward the cell contacts of galectin-1 on cancer cells (*A*) and of galectin-3 on endothelial cells (*B*). The superimposed fluorescent photomicrographs of DiI-labeled MDA-MB-435 breast cancer cells adhered to the monolayer of HUVEC cells (*C–E*). There is an inhibition of the adhesion by 0.1 mg/ml of the T antigen-specific synthetic P-30 (*E*) but not by the same concentration of the control peptide (*D*) compared with the control (*C*).

cells to human bone marrow endothelial cells (27). Thus, it was of interest to analyze whether β-galactoside-specific lectins participate in adhesion of the MDA-MB-435 cells to the endothelium. Confocal laser microscopy revealed the clustering of both galectin-1 and galectin-3 to the sites of contact between MDA-MB-435 cells and human umbilical endothelial cells (Fig. 3, *A* and *B*) indicative of their involvement in the interaction between cancer and endothelial cells. We could not observe, however, any sign of galectin-4 participation in this process, which was consistent with the data on its low level of expression in MDA-MB-435 cells.

Interestingly, galectin-1 and galectin-3 reacted differently on tumor and endothelial cells. A strong galectin-1 signal accumulated at the sites of tumor-endothelial cell contact predominantly on the cancer cells (Fig. 3*A*), which suggested the involvement of one or more of its cognate ligands on the endothelium. Galectin-3, in contrast, although also being strongly expressed on the tumor cells, clearly demonstrated signal accumulation toward the sites of the cell contact on HUVEC (Fig. 3*B*) possibly interacting with T antigen or other putative ligands on cancer cells. We hypothesized that if galectin-3 on the endothelial cells interacts with T antigen on MDA-MB-435 cells, then T antigen-specific P-30 should inhibit this interaction as it did in the case of homotypic aggregation. Thus, we performed experiments in which cancer cells were allowed to adhere to a monolayer of endothelial cells in the presence of P-30 (0.1 mg/ml final concentration) or a control peptide of identical concentration. The results of these experiments (Fig. 3, *C–E*) showed that the control peptide did not effect the adhesion of the MDA-MB-435 cells to the endothelial cells (Fig. 3, *C* and *D*), whereas T antigen-specific P-30 significantly (2-fold) inhibited it (Fig. 3*E*). When adhesion experiments were performed with different concentrations of P-30, we found the peptide's effect to be dose-dependent with the maximal inhibition achieved up to 50% (Fig. 4). These data demonstrated that adhesion of the MDA-MB-435 human breast carcinoma cells to the endothelial cells was, at least in part, mediated by T antigen. We observed the same inhibitory effects of the P-30 peptide on both adhesion to the endothelium and spontaneous homotypic aggregation of DU-145 human prostate carcinoma cells (data not shown), which suggests that similar molecular mech-

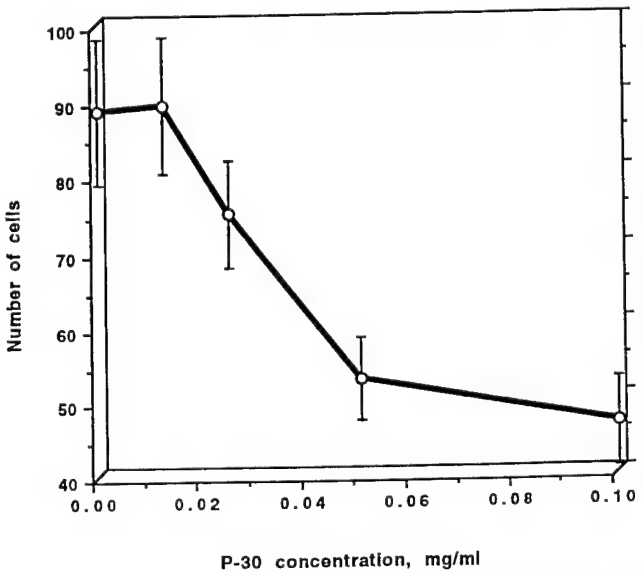

Fig. 4. Dose-dependent inhibition of the adhesion of MDA-MB-435 human breast carcinoma cells to the endothelium by synthetic T antigen-specific peptide P-30. The maximum inhibitory effect (about 50%) was achieved at 0.1 mg/ml concentration of the peptide. Representative results from one of three similar experiments are presented as mean of quadruplicate determinations. *Bars*, SD.

anisms of adhesion could be involved in different human malignancies.

Multicellular aggregate formation and adhesion of tumor cells to the endothelium are crucial events during early stages of cancer metastasis. Taken together, our data indicate that β-galactoside and particularly T antigen-mediated cell-cell interactions are important components of these events. To the best of our knowledge, this is the first observation that directly shows the accumulation of galectin-1 and galectin-3 at sites of contact between cancer and endothelial cells, which is indicative of their active participation in the adhesion of tumor cells to the endothelium.

Strikingly different behavior of these two β-galactoside-specific lectins reflects the complexity of the adhesion process. The accumulation of galectin-1 at the sites of cell-cell contacts predominantly on cancer cells and galectin-3 on endothelial cells suggests that several of their cognate ligands may be simultaneously involved here on both tumor and endothelial cells. Inhibition of tumor cell adhesion by the T antigen-specific P-30 peptide, however, highlights an active role for this cell surface carbohydrate structure in cancer-endothelial cell interactions. Recent observations of Al-Mehdi *et al.* (7) indicate that hematogenous metastases arise from the endothelium-attached tumor cells, which makes them particularly vulnerable to intravascular drugs capable of disrupting cancer-endothelial cell interactions. The ability of a short synthetic peptide to effectively interfere with this line of intercellular communication may also be of functional significance for the development of new antiadhesive therapies of cancer metastasis.

Two other types of compounds that also target β-galactoside-mediated adhesion have already been proven to be effective inhibitors of cancer metastases *in vivo* (28, 29). Specifically, synthetic analogues of naturally occurring conjugates of carbohydrates and amino acids (glycoamines) were shown to inhibit up to 75% both the incidence and number of MDA-MB-435 human breast cancer metastases in nude mice experiments (28). Modified citrus pectin, as reported by Pienta *et al.* (29), was also demonstrated to be an effective inhibitor of B16-F1 murine melanoma lung colonization as well as MAT-LyLu Dunning rat prostate cancer metastasis. Both synthetic glycoamines and modified citrus pectin act through the interaction with β-galactoside-specific lectins, specifically galectin-3, presumably by mimicking corresponding glycoepitopes of the cell surface glycomacromolecules or circulating glycoproteins (30). It is reasonable to hypothesize that the development of molecules directed against appropriate carbohydrate structures may likewise lead to the development of new effective antiadhesive therapies of cancer metastases.

This suggests new approaches to the concept of antiadhesive therapy of cancer (reviewed in Ref. 31), originally developed by early pioneering works of Dr. R. Kerbel and colleagues (32–34) and Dr. A. Raz and colleagues [Meromsky *et al.* (4 and Inohara and Raz (25)]. Traditional approaches to such therapy would be to generate appropriate sugar-specific antibodies. The difficulties of raising highly specific antibodies against carbohydrate moieties, as well as of the large-scale production of such antibodies, are well known, however. The development of carbohydrate-specific synthetic peptides using combinatorial bacteriophage display libraries could be a valid complimentary approach.

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The Role of Thomsen-Friedenreich Antigen in Adhesion of Human Breast and Prostate Cancer Cells to the Endothelium[1]

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ABSTRACT

Interactions of metastatic cancer cells with vasculatory endothelium are critical during early stages of cancer metastasis. Understanding the molecular underpinnings of these interactions is essential for the development of new efficacious cancer therapies. Here we demonstrate that cancer-associated carbohydrate T antigen plays a leading role in docking breast and prostate cancer cells onto endothelium by specifically interacting with endothelium-expressed β-galactoside-binding protein, galectin-3. Importantly, T antigen-bearing glycoproteins are also capable of mobilizing galectin-3 to the surface of endothelial cells, thus priming them for harboring metastatic cancer cells. The T antigen-mediated, tumor-endothelial cell interactions could be efficiently disrupted using synthetic compounds either mimicking or masking this carbohydrate structure. High efficiency of T antigen-mimicking and T antigen-masking inhibitors of tumor cell adhesion warrants their further development into antiadhesive cancer therapeutics.

INTRODUCTION

Metastasis is a major fatal complication associated with human malignant disorders. Recent findings demonstrate that hematogeneous cancer metastases originate from intravascular growth of endothelium-attached rather than extravasated cancer cells (1), highlighting the key role of tumor-endothelial cell interactions in cancer metastasis. Understanding the molecular mechanisms of these interactions is crucial for the development of new efficacious cancer therapies. A broad array of adhesion molecules, such as carbohydrates, lectins, cadherins, and integrins, have been implicated in the adhesion of tumor cells to the vasculatory endothelium. This reflects a complexity of the adhesion process. Moreover, different adhesion molecules have been shown to participate at distinct stages in a multistep binding process (2). For example, selectins were shown to contribute to the initial contact of circulating cells with endothelium by inducing their rolling (3), whereas galectin-3 has been proposed to participate in docking of cancer cells on capillary endothelium (4), and integrins were demonstrated to play a role in the development of more stable attachment involving protein-protein interactions (3).

Among all of the variety of adhesion molecules, cell surface carbohydrate structures have been a focus of many investigative efforts into their roles in cancer cell adhesion and metastasis. Aberrations in cell surface carbohydrates, emblematic of malignant transformation, have been shown to facilitate tumor cell colonization and metastasis by effecting normal cell-cell interactions (5, 6). One of the most widely distributed cancer-associated cell surface carbohydrate moieties is the pancarcinoma T antigen[3] (7). T antigen is a simple mucin-type disaccharide (8), Galβ1–3GalNAc, expressed on the outer cell surfaces of T-cell lymphomas and most human carcinomas (7–9), including breast and prostate (10). The role for T antigen in tumor cell adhesion and metastasis has been proposed based on the existence of T antigen-mediated adhesion of highly metastatic murine lymphoma cells and hepatocytes (9). Recently, we demonstrated the participation of T antigen in human breast carcinoma cell adhesion to the endothelium (11). We also reported that a 15-amino acid T antigen-specific peptide, P-30 (HGRFILPWWYAFSPS), selected from a bacteriophage display library (12, 13), specifically and significantly (>50%) inhibited adhesion of human breast cancer cells to the endothelium (11). These results underscored the importance of T antigen-mediated interactions in breast cancer metastasis. However, the molecular mechanisms of T antigen-mediated adhesion as well as cognate physiological receptors for T antigen have not been identified.

On the basis of the fact that the terminal residue of T antigen is β-galactose, we investigated its potential interactions with galectin-3 (11), a M_r ~30,000 member of a family of soluble β-galactoside-specific lectins (14, 15). Although the physiological role of this carbohydrate-binding protein is still greatly debated, galectin-3 has been implicated in several distinct fundamental cellular processes such as pre-mRNA splicing (16), cell growth and differentiation (17), regulation of apoptosis (18, 19), and cell-cell recognition and adhesion (19–21). In several experimental systems, the expression of galectin-3 in cancer cells was associated with increased malignant and metastatic phenotype (22, 23). Moreover, preferential adhesion of PC-3 human prostate cancer cells to bone marrow endothelial cells was found to be at least in part galectin-3-dependent (4). Recently, we demonstrated the involvement of the endothelium-expressed galectin-3 in breast carcinoma-endothelial cell adhesion (11) supportive of a possible interaction of this β-galactoside-binding lectin with T antigen.

We hypothesized that if T antigen is indeed interacting with galectin-3 during tumor cell docking onto the endothelium, it could be a common molecular mechanism of cancer cell adhesion pertinent to a metastatic dissemination of a variety of T antigen-expressing human malignancies. Carbohydrate-lectin interactions are believed to take place during an initial reversible phase of cell adhesion that determines cell-cell recognition specificity (24, 25). The efficient specific blockage of these early binding events may significantly modify the outcome of the adhesion and affect the metastatic process in whole.

In this study, we demonstrate that two synthetic inhibitors of T antigen-mediated adhesion, the T antigen-binding P-30 peptide, and a sugar-based T antigen mimetic, lactulosyl-L-leucine, efficiently (43–56%) inhibit the adhesion of breast and prostate carcinoma cells to

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[3] The abbreviations used are: T antigen, Thomsen-Friedenreich antigen; HUVEC, human umbilical vein endothelial cell; HBMEC, human bone marrow endothelial cell; FBS, fetal bovine serum; PNA, peanut agglutinin; DAB, diaminobenzidine tetrahydrochloride; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine; ASF, asialofetuin; T-PAA, T antigen-polyacrylamide; T-HSA, T antigen-human serum albumin; lactulosyl-L-leucine, *N*-(1-deoxy-4-O-(β-D-galactopyranos-1-yl)-D-fructofuranos-1-yl)-(*S*)-2-amino-4-methylpentanoic acid.

HUVEC monolayer. Both compounds exhibit an even more pronounced effect on cancer cell adhesion to human bone marrow microvascular endothelium (74–82% and 69–70% inhibition for breast and prostate carcinoma cells, respectively). These results strongly suggest possible involvement of T antigen-mediated adhesive interactions in breast and prostate cancer metastatic bone disease. Both T antigen-masking P-30 and T antigen-mimicking lactulosyl-L-leucine display the same maximal inhibitory effect on adhesion of breast and prostate cancer cells as a highly specific anti-T antigen monoclonal antibody does. The results of inhibition ELISA experiments confirm the specificity of both synthetic inhibitors and demonstrate direct binding of the recombinant human galectin-3 to the protein-linked T antigen. Remarkably, endothelial cells exhibit rapid and marked increase in cell surface galectin-3 expression when treated with T antigen-bearing glycoproteins. This observation suggests a novel function for circulating T antigen-expressing glycoproteins such as cancer-associated mucin, which may act by priming capillary endothelium for harboring cancer cells.

The results presented in this paper define T antigen as one of the leading factors during early stages of breast and prostate cancer-endothelial cell interactions. We demonstrate that T antigen is acting both as a major cell surface carbohydrate ligand for galectin-3 on breast and prostate cancer cells and as a factor causing mobilization of galectin-3 to the outer membrane on endothelial cells. The significance of T antigen-mediated adhesion in breast and prostate cancer identifies T antigen-galectin-3 interactions as a valid target for development of new antiadhesive therapies of cancer metastases.

MATERIALS AND METHODS

Antibodies, Chemicals, and Reagents. The monoclonal anti-T antigen antibody was developed as described (26). The TIB-166 hybridoma, producing rat monoclonal anti-galectin-3 (anti-Mac-2) was purchased from American Type Culture Collection. Goat Texas Red-conjugated antirat antibody was purchased from Molecular Probes (Eugene, OR). The monoclonal antibody to irrelevant plant protein was kindly provided by the Cell and Immunobiology Core (University of Missouri, Columbia, MO). The *Escherichia coli* strains, expressing human galectin-3 from pET5 vector, were kindly provided by Dr. A. Raz (Wayne State University, Detroit, MI) and Dr. H. Leffler (Lund University, Lund, Sweden). Recombinant human galecin-3 was isolated and affinity purified as described (27). DiI was from Molecular Probes (Eugene, OR). Biotinylated T-PAA was from Glycotech Corp. (Rockville, MD). T-HSA was from Dextra Laboratories (Reading, United Kingdom). T antigen binding peptide P-30 (HGRFILPWWYAFSPS) and control peptide (RRLLFYKYVYKRYRAGKQRG) were chemically synthesized using FMOC-based chemistry and purified to homogeneity on a C-18 reverse-phase HPLC column (ISCO Corp.). All other chemicals and reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines and Cultures. The MDA-MB-435 human breast carcinoma cell line was kindly provided by Dr. J. Price (M. D. Anderson Cancer Center, Houston, TX). The DU-145 human prostate carcinoma cells were obtained from American Type Culture Collection (Rockville, MD). B16-F10, a highly metastatic variant of B16-F1 mouse melanoma, was kindly provided by Dr. I. Fidler (M. D. Anderson Cancer Center). The RPMI 1640 supplemented with L-glutamine, 10% FBS, sodium pyruvate, and nonessential amino acids, was used for all tumor cell lines.

HUVECs were purchased from Cascade Biologicals (Portland, OR). Basal Medium 200 (Cascade Biologicals) supplemented with low serum growth supplement containing FBS (final concentration, 2% v/v), hydrocortisone, human fibroblast growth factor, heparin, and human epidermal growth factor was used for HUVECs. The cells at population doublings of approximately 8–12 were used for the adhesion experiments.

HBMEC line HBMEC-60 was kindly provided by Dr. C. E. van der Schoot (University of Amsterdam, Amsterdam, the Netherlands). This cell line was developed by immortalization of HBMECs originally isolated from adult human bone marrow using the amphotrophic helper-free retrovirus pLXSN16 E6/E7 (28). The HBMEC-60 cells were shown to maintain their normal phenotype and adhesive properties, specifically the ability to bind hematopoietic progenitor cells (28). Basal Medium 200 (Cascade Biologicals) supplemented with 20% FBS and low serum growth supplement containing hydrocortisone, human fibroblast growth factor, heparin, and human epidermal growth factor was used for HBMEC-60. All cells were maintained on plastic as a monolayer culture in a humidified incubator in 5% CO_2/95% air at 37°C.

Adhesion to the Endothelium. The adhesion experiments were performed exactly as described (11). A single cell suspension of cancer cells prelabeled with 3 μg/ml solution of acridine orange or 5 μg/ml solution of DiI in RPMI 1640 (5×10^4 cells/chamber in 2.5 ml of complete media supplemented with various concentrations of the compound tested) was added to the monolayer of the endothelial cells grown to confluence directly on microscope slides using 4-well chamber slides (NalgeNunc, Naperville, IL). The chambers were sealed, and cells were allowed to adhere for 1 h at 37°C, after which the chambers were inverted and left upside-down for 30 min to allow sedimentation of nonadhered cells. After that, the medium was drained, samples were gently rinsed with PBS, fixed for 30 min in 2% formaldehyde solution in PBS, mounted under coverglass, and examined by fluorescence microscopy. Four random fields in each well were photographed at ×250, and the total number of adhered cells in every field was counted. The assay was performed in quadruplicate for each condition.

T Antigen Cell Binding Assay. The B16-F10 mouse melanoma cell line was selected for these experiments because it does not expresses T antigen (12), which could interfere with the assay, but is capable of binding T antigen, as reflected by its interaction with ASF (13). The cells were placed on a microscope slide, fixed for 30 min in 2% formaldehyde solution in PBS, and incubated for 1 h in PBS containing biotinylated T-PAA (final concentration, 20 μg/ml) with or without lactulosyl-L-leucine (final concentration, 2 mM) at room temperature. Cells were washed three times with PBS. Bound T-PAA was visualized using peroxidase-labeled streptavidin and DAB as a chromogenic substrate. After counterstaining with hematoxylin, samples were mounted under coverglass and analyzed microscopically at ×900.

Dot Blot Assay. Bovine ASF, bearing multiple T antigen epitopes, was immobilized on the nitrocellulose membrane (1 μg/spot). After blocking with 2% solution of BSA in TBS, samples were incubated for 1 h at room temperature with different concentrations of peroxidase-labeled T antigen-specific PNA lectin without (control) or with addition of 2 mM (final concentration) of lactulosyl-L-leucine or lactitol-L-leucine in 2% BSA solution in TBS. The membranes were washed three times for 10 min each time in TBS and developed with DAB.

Inhibition ELISA. T-HSA (250 ng/well) was preabsorbed to 96-well plates overnight, blocked with 1% solution of BSA in PBS, and incubated for 1 h with 10 μg/ml solution of purified galectin-3 (1 μg/well) in the presence of different concentrations of T antigen-specific P-30 peptide (0–50 μM) or lactulosyl-L-leucine (0–1 mM) at room temperature. Bound galectin-3 was detected by sequential incubation with rat anti-galectin-3 monoclonal antibody, alkaline phosphatase conjugated goat anti-rat IgG, and *p*-nitrophenyl phosphate, followed by reading the absorbance at 405 nm. The plates were washed three times with PBS between steps. Controls containing no galectin-3 were included as blanks. Data represent the results of at least three determinations for each condition. The inhibition percentage was calculated as a decrease in the mean net absorbance at 405 nm compared with control.

Cytochemistry, Laser Scanning Confocal Microscopy, and Immunofluorescence. The cytochemical analysis of T antigen was performed using PNA-horseradish peroxidase conjugate and subsequent color reaction with DAB.

The samples for the analysis of galectin-3 cellular distribution were prepared exactly as described in adhesion to the endothelium, except that cancer cells were not prelabeled, and samples were fixed and permeabilized in 4% formaldehyde solution in PBS for 24 h. The rat anti-galectin-3 and goat Texas Red-conjugated antirat antibodies were used to visualize galectin-3. The laser scanning confocal microscopy was performed on a Bio-Rad MRC 600 confocal system. The Z stacks were prepared by obtaining serial sections with 0.5 μm increments and analyzed in orthogonal projections (*Y*-*Z* and *X*-*Z* sections) using the MetaMorph Imaging System software (Universal Imaging, Hallis, NH).

To evaluate cell surface galectin-3 expression, live, nonfixed, and nonpermeabilized endothelial cells, grown to confluence directly on microscope slides, were incubated for 45 min at 37°C in complete medium supplemented

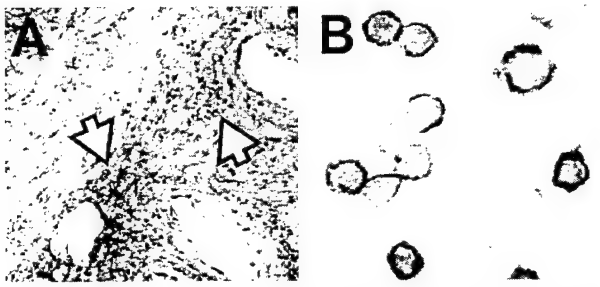

Fig. 1. Expression of T antigen in a human prostate cancer lesion (*A*) and in the DU-145 human prostate adenocarcinoma cell line (*B*) as revealed by binding of T antigen-specific PNA lectin-horseradish peroxidase conjugate followed by color reaction with diaminobenzidine tetrahydrochloride. *Arrows*, areas of a positive staining corresponding to the *brown color*, indicating the presence of immunoreactive T antigen.

with rat anti-galectin-3 antibody without (control) or with 1 mg/ml (final concentration) of bovine fetuin or ASF, or 0.5 mg/ml (final concentration) of HSA or T-HSA. Slides were washed three times with PBS and incubated for 1 h with goat Texas Red-conjugated antirat antibody. After additional washes, the samples were mounted under coverglass and analyzed on a Bio-Rad MRC 600 confocal system using accumulation of four scans. The four-well slides (NalgeNunc) were used, which allowed simultaneous processing of samples to be compared. The exact same conditions, such as iris settings, laser intensity, gain, magnification, and number of scan accumulations, were used for all samples.

RESULTS AND DISCUSSION

T Antigen Expression. The expression of T antigen in breast cancer tissues is well documented and is associated with tumor progression and metastasis (29). However, this carbohydrate structure is not specific only to breast cancer. Rather, it is characteristic of a vast majority of human adenocarcinomas. Similarly, T antigen is often detectable in prostate cancer lesions (Fig. 1*A*). Furthermore, in patients with prostate carcinoma, the expression of T antigen has also been found to correlate with tumor grade and metastasis (10, 30). Both breast (MDA-MB-435) and prostate (DU-145) cancer cell lines used in this study express T antigen on their surfaces. We demonstrated previously the presence of T antigen on MDA-MB-435 human breast carcinoma cells (13). This cell line, originally isolated from the pleural effusion of a patient with breast cancer, has been shown to be highly metastatic in nude mice (31, 32). The fact that adhesion of MDA-MB-435 cells to the endothelium could be efficiently (2-fold) inhibited by the T antigen-binding P-30 peptide (11) is indicative of the importance of T antigen in breast cancer-endothelial cell interactions. The DU-145 human prostate carcinoma cell line, chosen for our experiments, was also originally isolated from a metastatic lesion (33). Importantly, this cell line retains its metastatic potential, as reflected in the ability of DU-145 cells to develop metastasis in nude mice (34). In this study, we show that similar to MDA-MB-435 metastatic breast cancer cells, the DU-145 human prostate carcinoma cells also express T antigen on their surfaces (Fig. 1*B*). This observation suggests that T antigen might likewise participate in prostate cancer cell adhesion to the endothelium. To address this question, we investigated whether compounds, capable of specifically masking T antigen, would interfere with prostate cancer-endothelial cell interactions.

Inhibition of Cancer Cell Adhesion to HUVECs by Masking T Antigen. The T antigen-specific P-30 peptide (HGRFILPWWYAFSPS) was originally isolated from a bacteriophage display library (12). The synthetic P-30 binds with high affinity and specificity to both free T antigen disaccharide in solution and T antigen-bearing glycoproteins (13). It is also capable of specifically recognizing T antigen-expressing cancer cells of different origin (12, 13) and efficiently inhibiting ASF-mediated cancer cell aggregation (13) and breast carcinoma cell adhesion to the endothelium (11). Our previous results suggest that the peptide is masking T antigen epitopes on cancer cells, thus preventing interactions with their cognate ligands. In this study, we used the T antigen-masking P-30 peptide to investigate whether T antigen participates in prostate cancer cell adhesion to the endothelium. We studied the effect of different concentrations (0–0.1 mg/ml) of synthetic T antigen-specific P-30 on adhesion of DU-145 human prostate carcinoma cells to a monolayer of HUVECs. The results of these experiments (Fig. 2, *A–H*) showed that P-30 inhibited the adhesion of both DiI-labeled (Fig. 2*A*) and acridine orange-labeled (Fig. 2, *B–G*) DU-145 cells to endothelial cells in a dose-dependent manner, whereas the control peptide (RRLLFYKYVYKRYRAGKQRG) failed to inhibit adhesion (Fig. 2*H*). To assess the efficiency of T antigen masking by P-30 peptide, we compared the maximal inhibitory effects on breast and prostate cancer cell binding to the

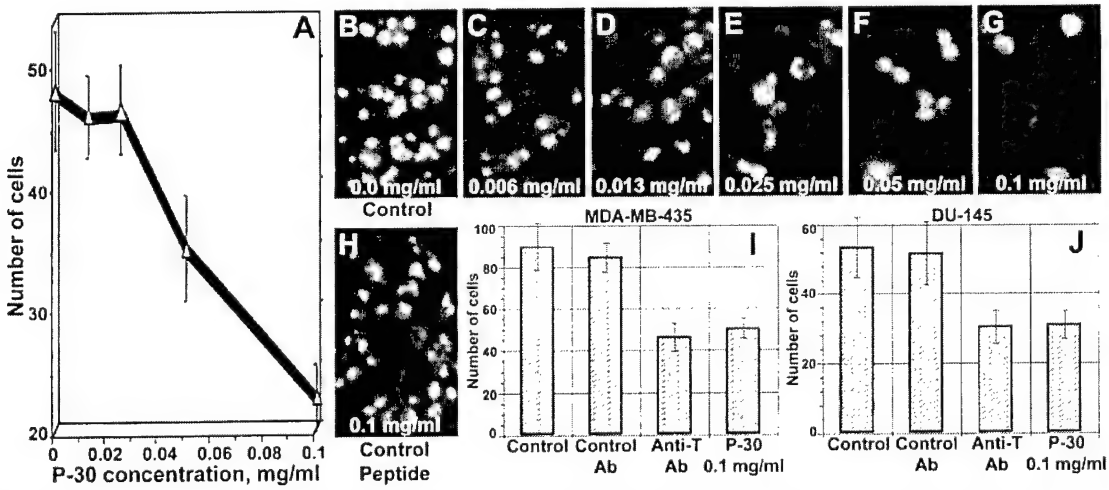

Fig. 2. Dose-dependent inhibition of DiI-labeled (*A*) and acridine orange-labeled (*B–G*) DU-145 human prostate carcinoma cell adhesion to the endothelium by synthetic T antigen-specific P-30 peptide but not by the control peptide (*H*). In *B–H*, numbers at the *bottom* indicate the concentration of the peptide tested. *I* and *J*, maximal inhibitory effect on adhesion of MDA-MB-435 human breast carcinoma (*I*) and DU-145 human prostate carcinoma (*J*) cells to the endothelium achievable with anti-T antigen monoclonal antibody and P-30 peptide; *bars*, SD.

endothelium, achievable with P-30 and an anti-T antigen antibody. A monoclonal antibody to irrelevant plant protein was used as a control in these experiments. We found that the control antibody did not inhibit attachment of tumor cells to HUVECs (Fig. 2, *I* and *J*), whereas both anti-T antigen antibody and P-30 exhibited almost identical maximal inhibitory effect on breast (Fig. 2*I*) and prostate (Fig. 2*J*) cancer cell adhesion. The results of these experiments demonstrated that synthetic P-30 peptide was masking T antigen as efficiently as the highly specific monoclonal anti-T antigen antibody. However, neither P-30 nor anti-T antigen antibody inhibited tumor cell adhesion to the endothelium completely. The inhibition efficiency (ranging from 43 to 56% in different experiments) displayed by these two compounds most likely reflected the impact of T antigen-mediated interactions in our experimental system. Apparently, different adhesion molecules such as integrins might also contribute to the binding. It has been suggested that circulating metastatic cells interact with endothelium in two distinct stages (24): the initial reversible docking stage, mediated by carbohydrate-lectin interactions; and the second stabilizing integrin-mediated locking stage, which requires more prolonged cell contact (25). In our adhesion experiments, cancer and endothelial cells were in physical contact for 1 h, allowing sufficient time for integrin-mediated binding events to occur. Supportive of this suggestion are results reported by Lehr and Pienta (4) using a similar experimental design. They demonstrated that antibodies to different members of the integrin family, such as CD11a, CD18, and leukocyte function antigen-1, inhibited adhesion of PC-3 prostate cancer cells to endothelium from 20 to 55%, suggesting the importance of integrins in stabilizing adhesion of tumor cells docked onto the endothelium. In contrast, interactions between T antigen and the corresponding carbohydrate-binding lectin(s) preceding integrin-mediated adhesion most likely are crucial during an initial docking stage. Supportive of this idea are the results of experiments using in-flow experimental systems,[4] showing that under conditions of flow, anti-integrin antibodies do not affect the adhesion of MDA-MB-435 breast carcinoma cells to the endothelium, whereas the synthetic T antigen mimetic lactulosyl-L-leucine inhibits it up to 80%. These results imply that T antigen, but not integrin-mediated interactions, plays a significant role in initiating cancer cell binding to the endothelium.

Inhibition of Cancer Cell Adhesion to HUVECs by Mimicking T Antigen. An independent approach to the study of T antigen-mediated adhesion would be to use compounds capable of mimicking T antigen. One such compound is a synthetic carbohydrate-amino acid conjugate (glycoamine), lactulosyl-L-leucine. Lactulosyl-L-leucine is capable of targeting β-galactoside-binding lectins (galectins) by mimicking the corresponding carbohydrate epitopes of naturally occurring glycoproteins (35). Similar to P-30, lactulosyl-L-leucine efficiently inhibits ASF-mediated cancer cell aggregation and competes with T antigen-specific PNA lectin for binding to breast carcinoma cells (36). In this study, we report that lactulosyl-L-leucine likewise inhibited the adhesion of breast (Fig. 3, *E* and *F*) and prostate (data not shown) tumor cells to the endothelium. The inhibition efficiency of this compound, 52 and 54% for breast and prostate carcinoma cells, respectively, was similar to that of the P-30 peptide and the anti-T antigen antibody. However, unlike P-30, lactulosyl-L-leucine is acting not through masking but through mimicking T antigen. The T antigen mimetic properties of lactulosyl-L-leucine are evident in its ability to compete off binding of T antigen to cancer cells (Fig. 3, *A–C*) and T

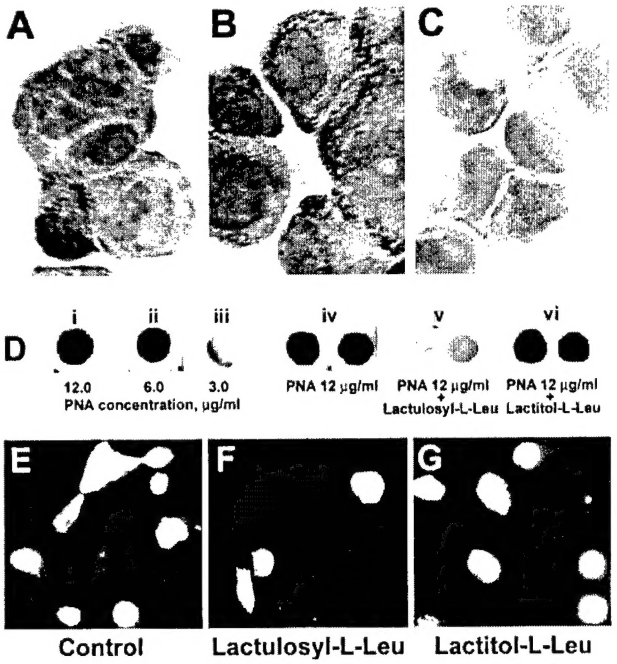

Fig. 3. T antigen-mimicking lactulosyl-L-leucine competes off biotinylated T antigen-PAA conjugate (*C*) bound to the B16-F10 mouse melanoma cells (*B*). *A*, control sample counterstained with hematoxylin. *D*, inhibition of T antigen-specific PNA lectin binding to ASF (*iv*) by T antigen-mimicking lactulosyl-L-leucine (*v*) but not by lactitol-L-leucine (*vi*); *i–iii*, binding of different concentrations of PNA lectin to ASF (1 μg) spotted on the nitrocellulose membrane. *E–G*, inhibition of DiI-labeled MDA-MB-435 breast carcinoma cell adhesion to the endothelium by T antigen-mimicking lactulosyl-L-leucine (*F*) but not by lactitol-L-leucine (*G*), compared with the control (*E*).

antigen-specific PNA lectin to ASF (Fig. 3*D*, *iv* and *v*). Interestingly, the substitution of lactulosyl for lactitol completely abolishes the ability of the resulting compound, lactitol-L-leucine, to both mimic T antigen (Fig. 3*D*, *vi*) and inhibit tumor cell adhesion to the endothelium (Fig. 3*G*). These results demonstrate that the antiadhesive properties of lactulosyl-L-leucine strictly depend on the carbohydrate moiety and aptitude of the compound to mimic T antigen. Importantly, this T antigen-mimicking compound was already shown to inhibit up to 75% both the incidence and number of spontaneous breast cancer lung metastases in nude mice experiments (36). This finding highlights the critical role of T antigen-mediated interactions in the metastatic process. In patients, however, both breast and prostate tumors most often metastasize to bone (37). Therefore, it will be very important to investigate whether T antigen is relevant to breast and prostate cancer metastatic bone disease.

Inhibition of Breast and Prostate Cancer Cell Adhesion to Human Bone Marrow Microvascular Endothelium. The results of postmortem examination demonstrate that ~70% of patients who die from carcinomas of the breast and prostate have evidence of bone metastases (37). Lately, it was suggested that specific tumor cell adhesive interactions with bone marrow endothelium could be an important factor in the predilection of prostate cancer metastases to the skeleton (4, 38). This hypothesis is strongly supported by the results presented recently by Lehr and Pienta (4). They demonstrated that prostate cancer cells adhere better to the bone marrow-derived endothelium than to the endothelium from other anatomical sites.

To further delineate the role of T antigen in breast and prostate cancer metastasis, we investigated whether T antigen participates in adhesion of MDA-MB-435 breast carcinoma and DU-145 prostate carcinoma cells to the monolayer of HBMEC-60 human bone marrow

[4] S. K. Khaldoyanidi, V. V. Glinsky, L. Sikora, A. B. Glinskii, V. V. Mossine, G. V. Glinsky, and P. Sriramarao. Role of galectin-3 in homo- and heterotypic intercellular adhesion of human metastatic breast carcinoma cells to endothelia under flow conditions, submitted for publication.

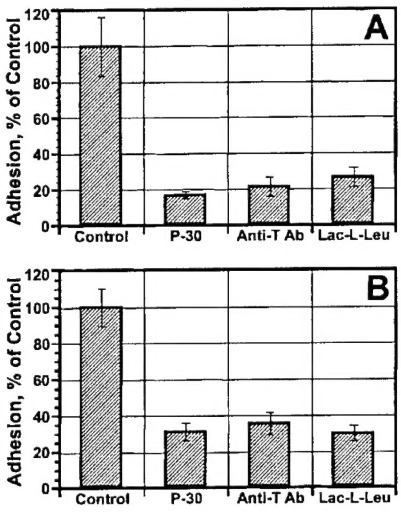

Fig. 4. Inhibition of MDA-MB-435 human breast carcinoma (*A*) and DU-145 human prostate carcinoma (*B*) cell adhesion to the monolayer of HBMEC-60 human bone marrow endothelial cells. The adhesion experiments were performed as described in "Materials and Methods" without (*Control*) or with 50 μM (final concentration) of T antigen-specific P-30 peptide, 1:2 dilution of anti-T antigen monoclonal antibody, or 1 mM (final concentration) of T antigen-mimicking lactulosyl-L-leucine. The adhesion was calculated as a percentage of control. The results of one of two identical experiments are shown; *bars*, SD.

endothelial cells. The results of these experiments (Fig. 4) show that both T antigen-masking (P-30 peptide and anti-T antigen antibody) and T antigen-mimicking (lactulosyl-L-leucine) compounds inhibit cancer cell adhesion to bone marrow endothelium even greater than to HUVECs (74–82% and 69–70% inhibition for breast and prostate carcinoma cells, respectively). These data indicate that T antigen-mediated interactions may play an important role in tumor cell adhesion to bone marrow microvasculature; however, the molecular basis of these interactions is still poorly understood. The identification of the molecules acting as T antigen receptors in cancer-endothelial cell adhesion will be of utmost interest.

Interaction of T Antigen with Galectin-3. Because the terminal sugar of T antigen is β-galactose, the involvement of β-galactoside-binding lectins (galectins) in T antigen-mediated adhesion was investigated. Two of nine currently known galectins, *i.e.*, galectin-1 and galectin-3, were found previously to be most prominently expressed in cancer and endothelial cells and were implicated in cancer cell adhesion (39–41). On the basis of the fact that human galectin-3 exhibits 200-fold higher specific activity toward Galβ1–3GalNAc disaccharide than galectin-1 (14), we proposed previously the interaction of T antigen with galectin-3 during cancer cell adhesion to the endothelium (11). Supportive of this interaction is the observation reported by Bresalier *et al.* (42) that colon cancer mucin, a glycoprotein most often decorated with multiple T antigen epitopes, serves as a specific ligand for galectin-3. Moreover, they also demonstrated that fully glycosylated mucin binds >40-fold more galectin-3 than mucin from the cells in which oligosaccharide synthesis is blocked at the stage of addition of β3-linked galactose to the *N*-acetylgalactosamine (Tn antigen) to form T antigen. We reasoned that if T antigen is in fact interacting with galectin-3, then both T antigen-masking and T antigen-mimicking compounds should specifically inhibit this interaction. To test this hypothesis, we performed inhibition ELISA experiments in which T antigen conjugated to HSA was preabsorbed on plastic in 96-well plates and incubated with purified recombinant human galectin-3 in the presence of different concentrations of the P-30 peptide or lactulosyl-L-leucine. We found that both compounds inhibited galectin-3 binding to T antigen-HSA conjugate in a dose-dependent manner (Fig. 5). Although the effect of both compounds was saturable, neither one reached 100% inhibition. We attribute the remaining binding (~30%) to cooperative interactions characteristic of galectin-3 (17). To the best of our knowledge, this is the first observation that directly demonstrated galectin-3 binding to protein-linked T antigen. These data also confirm the specificity of both inhibitors tested, T antigen-masking P-30 peptide and T antigen-mimicking lactulosyl-L-leucine.

The role of galectin-3 in prostate cancer cell adhesion was challenged recently, however, by the results reported by Ellerhorst *et al.* (2). They demonstrated that galectin-3 is not present on the cell surface in any of the four prostate cancer lines they tested, including DU-145, which was used in our experiments, and PC-3, which was shown by another group to bind to the endothelium in a galectin-3-dependent manner (4). To clarify this issue, we investigated cellular distribution of the galectin-3 during cancer-endothelial cell interaction.

Cellular Distribution of Galectin-3 during Cancer Cell Adhesion to the Endothelium. Laser scanning confocal microscopy was used to study the localization of the galectin-3 in both cancer and endothelial cells upon their interaction. This technique allowed us to analyze the orthogonal plans (*X-Z* and *Y-Z* sections), which included both cancer and endothelial cells, as well as the sites of their contact. The results of these experiments (Fig. 6, *A* and *B*) revealed remarkably similar patterns of galectin-3 distribution during DU-145 prostate carcinoma (Fig. 6*A*) and MDA-MB-435 breast carcinoma (Fig. 6*B*) cell adhesion to HUVECs. In both cancer cell lines, galectin-3, although expressed at higher levels than in endothelial cells, remained predominantly intracellular. This result is in agreement with observations of Ellerhorst *et al.* (2) and suggests that galectin-3 of cancer cells does not actively participate in their adhesion to the endothelium. In contrast, on endothelial cells we observed the mobilization and clustering of this carbohydrate-binding protein toward the cancer-endothelial cell contacts. This phenomenon is consistent with the interaction of endothelium-expressed galectin-3 with T antigen displayed on the surface of cancer cells and is indicative of its active role in cancer-endothelial cell adhesion. These results also provide a feasible

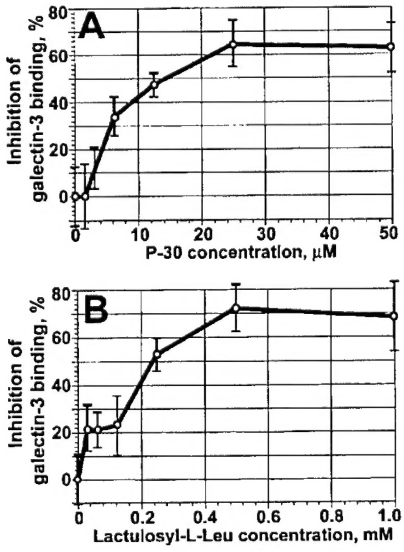

Fig. 5. Dose-dependent inhibition of recombinant human galectin-3 binding to T antigen-HSA by T antigen-masking P-30 peptide and T antigen-mimicking lactulosyl-L-leucine. The inhibition percentage was calculated as a decrease in the mean net absorbance at 405 nm compared with the control; *bars*, SD.

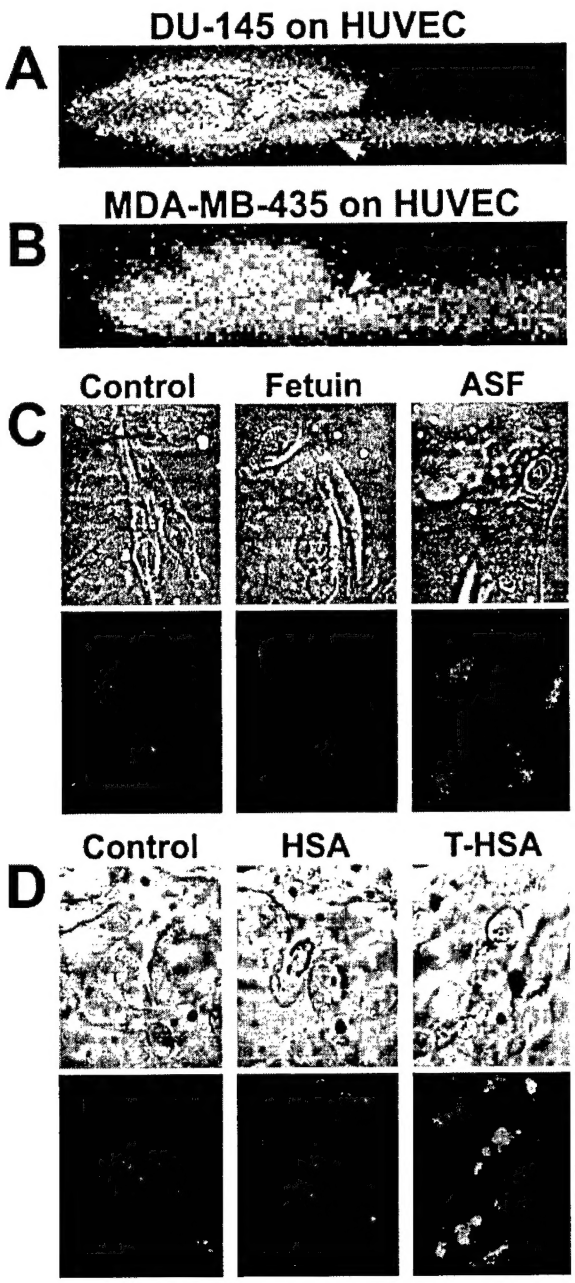

Fig. 6. *A and B*, pseudocolored *X-Z* sections showing cellular distribution of galectin-3 upon adhesion of DU-145 prostate (*A*) and MDA-MB-435 breast (*B*) cancer cells to the endothelium. Different colors represent different concentrations of galectin-3 (*purple, blue, green, yellow, red,* and *white,* from lowest to highest). Note the accumulation and clustering of galectin-3 toward cell contacts on endothelial cells indicated by *arrows*, whereas in cancer cells the protein remains predominantly intracellular. *C* and *D*, the effect of ASF and T-HSA on galectin-3 surface expression on endothelial cells. Note the increase in galectin-3 surface representation upon treatment with ASF (*C*) or T-HSA (*D*) but not with fetuin or HSA, compared with control. In both *C* and *D*, the *top panel* shows a corresponding transmitted light photomicrograph of the same field.

explanation of why PC-3 cells, which do not exhibit galectin-3 on a cell surface (2), interact with endothelium in a galectin-3-dependent manner, as reported by Lehr and Pienta (4).

The fact that endothelium-expressed galectin-3 clustered toward the sites of cancer-endothelial cell contacts obviously attracted our attention. Thus, we decided to investigate whether T antigen was a factor causing galectin-3 mobilization and clustering. We studied the changes in galectin-3 surface expression on endothelial cells after treatment with T antigen-bearing glycoproteins, ASF and T-HSA. Both ASF and T-HSA display multiple immunoreactive T antigen epitopes. Two other proteins, bovine fetuin and HSA were used as corresponding negative controls in these experiments. Bovine fetuin is identical to ASF and also contains multiple T antigen antennas, which are, however, covalently masked with sialic (neuraminic) acid and nonimmunoreactive. HSA does not contain T antigen in any form. These experiments revealed that treatment with either ASF (Fig. 6*C*) or T-HSA (Fig. 6*D*) resulted in a rapid and significant increase in galectin-3 expression on a surface of endothelial cells, whereas neither fetuin nor HSA effected this expression. These results demonstrate that T antigen is acting not only as a ligand for galectin-3 but also as a factor causing the mobilization of this carbohydrate-binding protein to the cell surface. The latter observation in turn suggests an important novel function for the circulating cancer mucin, which is often found in the serum of patients with adenocarcinomas of different origin (42, 43). Circulating cancer mucin, similar to ASF and T-HSA, bears multiple T antigen moieties and may likewise induce the increase in galectin-3 surface expression on endothelial cells, thus priming vasculatory endothelium for binding the circulating metastatic tumor cells. Importantly, this phenomenon could be observed when highly metastatic T antigen-expressing MDA-MB-435 cells interact with microvascular endothelium under conditions of flow.[4] In contrast, the MDA-MB-468 cells, which are deficient in T antigen expression and are nonmetastatic, do not induce galectin-3 surface mobilization. This observation implies that T antigen-expressing circulating metastatic cancer cells could themselves change endothelium adhesiveness.

The fact that T antigen can actually modify the adhesive properties of the endothelium by mobilizing cell surface adhesion molecules such as galectin-3 adds new and important insights into our understanding of tumor-endothelial cell interactions. It suggests the existence of an additional priming stage in adhesion of circulating metastatic cells to the endothelium, which precedes the docking (initial reversible adhesion) and locking (permanent irreversible adhesion) stages (Fig. 7).

The results presented in this report identify T antigen as a major cancer cell surface carbohydrate ligand for galectin-3. Although both the former and the latter may also interact with other cognate ligands, the T antigen-galectin-3 interactions are likely to play a leading role in the initial stages of cancer cell adhesion to the endothelium. The efficient intervention with cancer cell endothelium interactions could result in the development of new antiadhesive cancer therapies (reviewed in Ref. 44), the concepts of which were brought about by early pioneering works of R. Kerbel *et al.* (45, 46) and Inohara and Raz

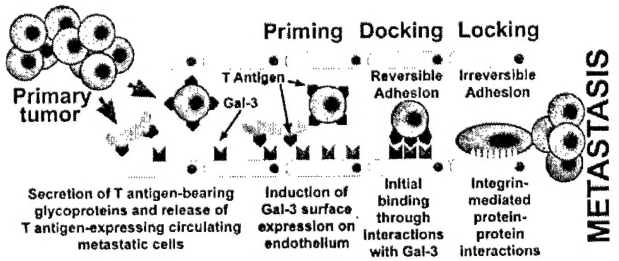

Fig. 7. Schematic representation of T antigen participation in adhesion of metastatic cancer cells to the vasculatory endothelium. Circulating T antigen-bearing cancer-associated glycoproteins and metastatic tumor cells induce the mobilization of the galectin-3 to the surface of endothelial cells (*Priming* stage). T antigen-expressing malignant cells bind to the endothelium through T antigen-galectin-3 interactions (*Docking*) allowing sufficient time for the stabilizing integrin-mediated adhesion events to take place (*Locking* stage).

(47). The significant inhibition of breast and prostate cancer cell adhesion to the endothelium by T antigen-masking and T antigen-mimicking compounds suggests their high potential to be developed into efficacious antimetastatic agents.

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